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13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the last year of the project, we have carried out the proteomic analysis of epidermal extracts from the NF-1 null and wild type skin. This analysis validated the results obtained when using extracts from cultured keratinocytes for the interacting partners of NF-1. Initial experiments using lysates from primary mouse keratinocytes cultured in vivo from both wild type and knockout mice further confirmed the interactions suggested by the proteomic analyses. In relation to the development of psoriasis-like symptoms in the NF1 null epidermis, we analyzed NF1 expression in a mouse model of psoriasis (imiquimod-induced psoriasis-like skin inflammation) and documented a decrease in NF1 expression levels. Consequently, we have obtained in vivo data from multiple systems supporting our use of the conditional knockout of epidermal NF1 to elucidate the molecular underpinnings of psoriasis.

15. SUBJECT TERMS

neurofibromin-1 (NF1), psoriasis, inflammation, skin

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1. Introduction

The loss of murine homolog NF1 in mice leads to embryonic lethality at E13.5 because of abnormal cardiac development. Thus, to study the function of NF1 in a somatic tissue such as the epidermis, we generated epidermal knockout mice of NF1 (NF1 cKO) by crossing of mice harboring $Nf1^{flox}$ alleles with the K14-Cre transgenic mice. The epidermis is a stratified epithelia, and the basal layer is the proliferating layer of the tissue, where the cells maintain the ability of self-renew and facilitate tissue regeneration. Accumulating data demonstrate that constitutively activated Ras leads to hyper-proliferation both in vivo and in vitro. Interestingly, the deletion of NF1 from the basal layer of the epidermis did not cause the hyperplasia of the basal layer as we expected. Instead, we saw the expansion of the spinous layer, which is composed of differentiated keratinocytes. Because microtubules play critical roles in regulating asymmetrical cell division and stratification of epidermis and NF1 localizes to microtubules (MTs) in many cell lines including keratinocytes [16-19], NF1 may regulate the MTs and skin stratification. Moreover, 8-month-old NF1 cKO develop a psoriasis related inflammatory response. In both heterozygous and homozygous mice with NF1 mutation, we detected hyper-activation of Ras by immunohistochemistry staining and western blotting of phospho-MAPK (pMAPK), the major down-stream indicator of activated Ras (data not shown). Despite the elevated Ras activity, the epidermal phenotypes were only observed in the knockout but not the heterozygous mice. Thus, the epidermal phenotypes of NF1 cKO cannot be simply explained by different dosage of Ras activity in homozygous and heterozygous NF1 mutant mice. Overall, the loss of NF1 in epidermis provides a unique model system to uncover novel Ras-independent functions of NF1.

2. Keywords

Neurofibromin-1 (NF1), epidermis, corneodesmin (CDSN), Dihydropyrimidinase-related protein, psoriasis, spindle orientation, microtubules, GAIP-interacting protein C terminus 1

3. Accomplishments

What were the major goals of the project?

The specific aims of this project are:

1. Perform mass spectrometry analysis of NF1 complex in skin extracts to uncover NF1 binding partners under physiological conditions
2. Dissect the role of NF1 in regulating microtubule function through Dpysl2 and Dpysl3.
3. Examine if loss of NF1 in the epidermis results in the malfunction of CDSN and the development of psoriasis-like symptoms.
4. Investigate if GIPC1 is a potential drug target for Nf1 treatment

What was accomplished under these goals?

The activities, objectives, results and other achievements will be listed according to the specific aims:

<u>1. Uncover NF1 interacting proteins</u>

The initial screen of interacting partners of NF1 was performed on cultured mouse keratinocytes. Given the differences between the activity of various proteins in vitro and in vivo, we validated whether the immunoprecipitation of NF1 yields the same interacting partners in a lysate made from whole skin. Solubilization of the skin (and in particular the NF1 containing fraction) required substantial optimization as protein degradation and low yield was a frequent obstacle.

Changes in buffer conditions and detergents helped in overcoming this problem and we were able to isolate enough NF1 complexes to perform mass spectrometry. Initial analysis of the data validates the proteins identified as NF1 interacting partners in cultured keratinocytes. There were other interacting partners identified using whole skin lysates, but at this time we are not sure if this interaction occurs in the epidermal keratinocytes or any of the other cells that populate the skin (such as dermal fibroblasts, sebocytes, nerve cells, and endothelial cells). Optimization of the immunoprecipitation protocol using NF1 antibodies will facilitate our immediate goals of performing the reciprocal precipitation using antibodies against the interacting partner to validate the proteomic data.

2. Role of Dpsyl2 and 3 in NF1 mediated regulation of microtubules

The major effort in this aim was focused on generating the mice lacking epidermal NF1 with labeled microtubules (K14-Cre, $NF1^{fl/fl}$, K14-EMTB-GFP) and its corresponding control (K14-Cre, $NF1^{+/fl}$, K14-EMTB-GFP). The mice are almost ready to analyze to probe the organization of microtubules in vivo in the presence or absence of NF1. Moreover, primary keratinocytes harboring these genotypes will be isolated and cultured to probe the impact of NF1 and Dpsyl 2 & 3 on microtubule organization.

3. Examine if loss of NF1 in the epidermis results in the malfunction of CDSN and the development of psoriasis-like symptoms.

The bulk of our efforts over the past year have been focused on this aim. In particular, we have been analyzing the ability of the NF1 conditional knockout mouse to recapitulate the features of psoriasis and thus serve as a model to understand the pathophysiology of this disease. To this end we have analyzed the expression of NF1 in the imiquimod treated mice, which is a well-established mouse model of psoriasis-like skin inflammation. Interestingly, NF1 expression is decreased 50% in the imiquimod treated mice relative to vehicle treated animals (Figure 1). This reduction in NF1 expression is not a generic response to inflammation as a Snail transgenic mouse with robust cutaneous inflammation that serves as a model for the fibrotic skin disease scleroderma does not exhibit any reduction in NF1 mRNA levels (Figure 2). Moreover, corneodesmin (CDSN) is also not reduced in the Snail transgenic skin (Figure 3), but preliminary data suggests that CDSN is significantly reduced in the NF1 conditional knockout and imiquimod treated skins (data not shown).

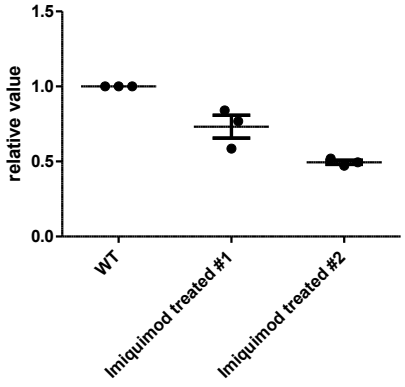

Figure 1. Expression of NF1 in the skin of mice treated with imiquimod to elicit psoriasis-like skin inflammation. Two mice were treated with imiquimod and one mouse was treated with vehicle control (WT). The levels of NF1 were determined by qPCR and the level of NF1 in the WT mouse was normalized to 1.

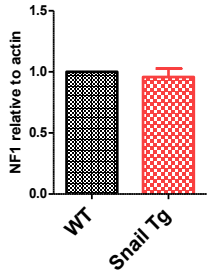

Figure 2. Expression of NF1 in the skin of another mouse model of inflammation. Skin from Snail transgenic mice (Snail Tg) mice that show inflammation and features of the skin disease scleroderma were analyzed for NF1 expression via qPCR. The levels of NF1 in the WT mouse was normalized to 1.

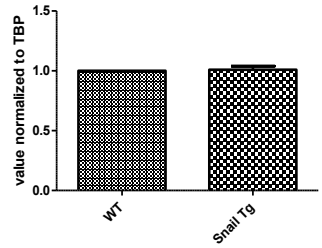

Figure 3. Expression of corneodesmin (CDSN) in Snail transgenic skin

4. Investigate if GIPC1 is a potential drug target for Nf1 treatment

We have not yet initiated experiments on this aim. Since this aim will follow the workflow of the aims described above, we focused on optimizing these commonly used assays. Now that these assays are reproducible, work on this aim will commence in the upcoming funding year.

What opportunities for training and professional development has the project provided?

The project supported a postdoctoral fellow with aspirations to be on academic research. The project helped the fellow to get trained in various in vitro and in vivo studies such as proteomic analyses and skin explant cultures.

How were the results disseminated to communities of interest?

Nothing to report at this time

What do you plan to do during the next reporting period to accomplish the goals?

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

The mouse lacking NF1 in the epidermis has the potential of shedding important new insights into the pathogenesis of psoriasis and possible new therapeutic targets. On the basic science front, this work will advance our understanding of tissue morphogenesis and homeostasis in the context of controlling differentiation of progenitor cells.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report; however, findings from the study could aid new drug designs for psoriasis, a common skin condition.

5. Changes/Problems
Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
We have encountered problems with identifying and using antibodies recognizing proteins described in the grant that have delayed the progress towards achieving the specific aims. Many of the commercial antibodies that we have purchased were not able to provide convincing staining despite numerous attempts at optimization. We have therefore abandoned commercial sources of these antibodies and requested them from other academics who have generated these reagents in their own labs. Based on our discussions with them we are confident that these source of antibodies will be functional in our assays.

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals.
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

6. Products
Publications, conference papers, and presentations
Nothing to report

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Development of potential new mouse model of psoriasis based on the conditional knockout of epidermal NF1.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name	Shyni Varghese
Project Role	PI
Research Identifier	svarghese
Nearest Person Month Worked	1
Contribution to Project	Dr. Varghese worked with Dr. Shih and others to formulate the experiments and analyze the data.
Funding Support	NIH/CIRM/DoD

Name	Yuru (Vernon) Shih
Project Role	Postdoctoral Fellow
Research Identifier	YVSHIH
Nearest Person Month Worked	12
Contribution to Project	Dr. Shi has performed the experimental work
Funding Support	DoD/NIH

- *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*
  Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

NO

What other organizations were involved as partners?

No

8. Special Reporting Requirements
Collaborative awards
Nothing to report

9. Appendices……
None